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Kinetic and epitope characterization of influenza antiviral targets using Alto™ digital surface plasmon resonance

Summary

Rapid changes in influenza antiviral target proteins due to antigenic drift result in cloaking of the influenza virus from the immune system of vaccinated hosts. Hence, annual formulation updates for influenza vaccines and related antibody therapies are required to preserve immune recognition against different influenza subtypes. As such, characterizing the binding kinetics and epitope diversity of various antibodies to influenza viral antigens is essential for treating and preventing potential outbreaks. In collaboration with Sino Biological, a global leader in recombinant technology, we use Nicoya's Alto digital surface plasmon resonance (SPR) system to perform kinetic analysis and epitope characterization of several antibodies against Influenza A nucleoprotein (NP) and hemagglutinin (HA), using only 2 μL per sample.

Introduction

Influenza, or "the flu", is a contagious viral infection caused by the influenza virus. Of the four types (Influenza A, B, C, and D), the two main flu viruses impacting humans are Influenza A and B, both being highly contagious and routinely causing seasonal epidemics. Furthermore, Influenza A viruses are the only known to cause flu pandemics, with all five historical instances of flu pandemics since 1900 being associated with Influenza A.

With the constant evolution of viruses, binding and characterization studies of viral antibodies and antigens are critical to managing and preventing future viral outbreaks. In addition, these studies advance our understanding of viral antigenic drift and diversity of flu-related antibodies.

Influenza A Nucleoprotein (NP)

Influenza viral nucleoprotein is a structural protein that plays a critical role in viral replication and host adaptation. Antibodies targeting NP proteins are commonly used for immunodetection of influenza viruses in various assays, including enzyme linked immunosorbent assays (ELISA), lateral flow assays (LFA), and direct fluorescent antibody tests. Due to the high frequency of antigenic drift or shift among different influenza strains, broad-spectrum influenza antibodies are particularly desired for flu diagnostics.

Influenza A Hemagglutinin (HA)

The HA protein receptor-binding domain (RBD) is critical for vaccine development and a popular target for antibodies due to their ability to directly inhibit binding of the virus to the host cell receptor. In addition, changes in this protein are essential for continual development of vaccines.

Traditional Characterization Techniques

Traditional techniques such as ELISA and western blot (WB) require time-consuming washing and incubation steps, and depend on the use of tags for analysis. Compared to such traditional techniques, the information content provided by SPR enables increased efficiency in the development of viral diagnostics and therapeutics. SPR provides complete kinetic characterization of antibody-antigen interactions to understand, rank, and optimize antibody therapeutics, without the use of tags or labels. In addition, it offers the unique advantage of epitope binning capabilities to better understand epitope diversity and overlap.

Kinetic Analysis and Epitope Characterization with Alto

Alto provides digital microfluidic (DMF) powered SPR with an unprecedented ability to study, develop and optimize vaccines & antibody therapeutics among many other applications. Full kinetic characterization with SPR

enables a deeper understanding of both antibody efficacy and antigen drift. Alto accelerates analysis with sample volumes as low as 2 µL and the execution of automated on-cartridge serial dilutions with this volume.

Sino Biological provides recombinant antigen products for all WHO-recommended vaccine strains including HA, NA and nucleoproteins for use in SPR-based assays. In collaboration with Sino, this application note features kinetic and epitope characterization studies conducted on Alto with five unique antibodies against two influenza A NP proteins, and two unique antibodies against an influenza A HA protein.

Material and Equipment

- Alto Instrument (ALTO16)

- Alto CBX Cartridge (KIN-CART-CBX-16)

- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)

- Regeneration Buffer: 10mM Glycine-HCl pH 2.0 (ALTO-R-GLYHCl-2.0)

- Nicoya Amine Coupling Kit (ALTO-R-EDCNHS)

- Ligands and analytes: All viral products were supplied by Sino Biological. For a full list of products and their Catalog #, please see Appendix.

Method

Direct Kinetics

Experimental setup was remotely completed on Alto's User Portal, followed by run initiation on the instrument:

1. From a laptop, the experiment (Run Method) was designed and saved on the User Portal.

2. On the instrument, the designed experiment was selected to launch Alto's on-screen setup wizard.

3. A Carboxyl Cartridge was loaded and all reagents were pipetted according to the wizard.

4. The experiment was initiated by selecting the 'Run Method' command.

The remainder of the experiment was executed automatically by Alto, with the operator being completely hands-off:

5. Carboxyl sensors were primed with 10 mM Glycine-HCl for 40 s.

6. Carboxyl surface was activated with EDC/NHS for 5 min.

7. The Influenza A Antibody ligands at 20 µg/ml in acetate pH 5.0 were immobilized for 10 min on each of the active sensors.

8. All sensors were blocked using an ethanolamine blocking solution for 5 min to quench the remaining active Carboxyl groups.

9. Sensors were incubated in PBS-T for 15 min to collect blank injection curves.

10. The experiments were performed using both single-cycle kinetics (SCK) and multi-cycle kinetics (MCK) on two separate cartridges:

    a. Single-cycle kinetics: Alto executed five automated 3-fold serial dilutions on the cartridge per Influenza A Protein analyte sample from the 300 nM stock concentration, producing 1.2 nM, 3.7 nM, 11 nM, 33 nM and 100 nM analyte samples. Influenza A Protein analyte samples were introduced in increasing concentrations with an association time of 180 s, without dissociation or regeneration between each sample, starting from the lowest concentration. The last (highest) analyte concentration was followed by a 1200 s dissociation. The sensor surface was regenerated with a 60 s exposure of Glycine-HCl pH 2.0.

    b. Multi-cycle kinetics: Alto executed five automated 3-fold serial dilutions on the cartridge per Influenza A Protein analyte sample from the 600 nM stock concentration, producing 2.5 nM, 7.4 nM, 22.2 nM, 66.7 nM and 200 nM analyte samples. First concentration of the Influenza A Protein analyte sample was introduced with an association time of 180 s, followed by a 1200 s dissociation and a 60 s regeneration of Glycine-HCl pH 2.0. This was repeated for the next 4 concentrations.

Data analysis:

11. Upon completion of the test, all binding curves were fitted to a 1:1 binding model to determine kinetic and affinity constants.

Pan Influenza A Nucleoprotein Antibody Binning

1. Steps 1 through 10 above were followed to directly couple each one of the Pan Influenza A Nucleoprotein Antibodies to an active sensor for use as the surface antibody.

2. Influenza A Protein antigen (100 nM) was introduced to each capture antibody.

3. Alto executed five automated 3-fold serial dilutions on cartridge per Pan Influenza A Nucleoprotein Antibody used as the "secondary antibody" sample from the 300 nM stock concentration, producing 1.2 nM, 3.7 nM, 11 nM, 33 nM and 100 nM analyte samples.

4. The sensor surface was regenerated with a 60 s exposure of Glycine-HCl pH 2.0 to remove the secondary antibody and the antigen.

5. Steps 2 through 4 were repeated for each additional Pan Influenza A Nucleoprotein Antibody.

6. Completion of test. Secondary antibody was unable to bind to the captured antigen if it competed for the same or similar epitope as the surface antibody.

Results and Discussion

Kinetic Characterization

The binding and kinetic fits of the Influenza A Proteins to the immobilized Influenza A Antibodies resulted in good binding curves and kinetic parameter evaluations. Examples of the resulting SCK and MCK overlays are presented in Figures 1 and 2, respectively.

The data was fit to a one-to-one binding model and the calculated kinetic constants are presented in Table 1. The kinetic data shows high affinity for the three Influenza A Proteins to all of the Influenza A Antibodies tested.
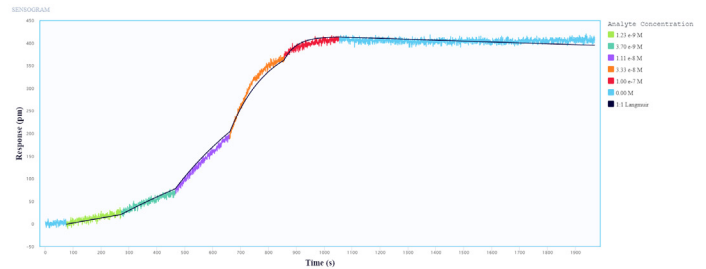

Figure 1: *Single-cycle kinetics of NP 2 antigen (analyte) binding to immobilized NP III antibody (ligand) on Alto. Analyte was titrated from 1.2 nM to 100 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.*
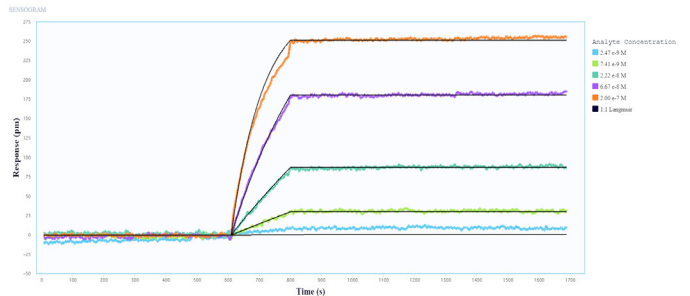

Figure 2: *Multi-cycle kinetics of HA antigen (analyte) binding to immobilized HA I antibody (ligand) on Alto. Analyte was titrated from 2.4 nM to 200 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.*

Table 1: Kinetic values measured using Alto data with the Nicoya analysis software.

Pan Influenza A Nucleoprotein	Antigen NP 1 40205-V08B				Antigen NP 2 40208-V08B			
Antibody	K_a (1/(M*s))	k_d (1/s)*	K_D (M)	Volume (µL)	K_a (1/(M*s))	k_d (1/s)*	K_D (M)	Volume (µL)
NP I	8.18E+04	1.55E-05	3.73E-10	2	2.59E+04	1.00E-06	4.55E-11	2
NP II	5.89E+04	3.18E-06	2.47E-10	2	2.15E+04	1.12E-05	5.21E-10	2
NP III	3.88E+04	1.00E-06	5.90E-11	2	3.42E+04	1.00E-06	4.97E-11	2
NP IV	5.78E+04	2.00E-06	3.27E-11	2	4.66E+04	1.00E-06	3.77E-11	2
NP V	3.66E+04	9.15E-05	1.11E-09	2	2.97E+04	1.00E-06	2.46E-11	2

Influenza A H3N2 Hemagglutinin	Antigen HA 11056-V08H			
Antibody	K_a (1/(M*s))	k_d (1/s)	K_D (M)	Volume (µL)
HA I	6.41E+04	1.00E-06	2.36E-11	2
HA II	6.23E+04	1.00E-06	9.67E-11	2

* Note that Alto instrument has a lowest K_d limit of 1E-6 1/s.

Epitope Characterization

The epitope diversity of Sino Biological's reagents were evaluated with a sandwich assay utilizing a Capture methodology. Figures 3 and 4 show the results of a 5x5 epitope bin with "Bind" results signifying a unique epitope targeted by the antibodies. In addition to epitope characterization, in the same experiment, kinetic values corresponding to each antibody were also obtained with on-cartridge serial dilutions.
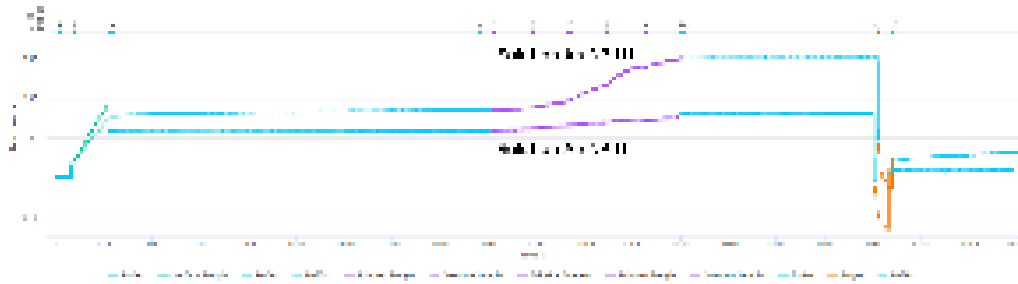
Figure 3: *Epitope characterization showing capturing of the Influenza A NP Protein antigen (green curve) to the immobilized surface antibody NP I followed by binding of the solution antibody to a different epitope of the antigen (purple - NP III). A lack of binding of the solution antibody in the purple - NP II curve indicates epitope overlap of NP II with NP I. Full regeneration is achieved by 10 mM glycine HCl pH 2.0 (orange curve returning the response to the baseline before antigen capture).*

Antigen: Influenza A Nucleoprotein (NP): SinoBiological Cat # 40205-V08B

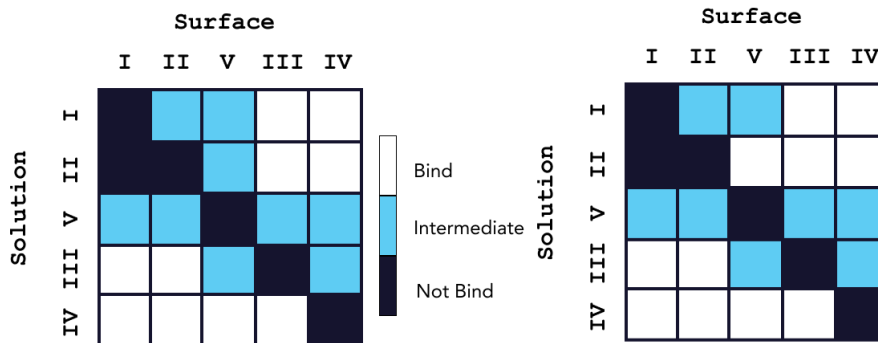Antigen: Influenza A Nucleoprotein (NP): SinoBiological Cat # 40208-V08B

Figure 4: *Epitope characterization analysis for Pan Influenza A Nucleoprotein Antibody binding to Influenza A Nucleoprotein Proteins Cat: 40205-V08B and 40208-V08B. The degree of binding displayed in the heat map was determined based on the change in response levels after the addition of the solution antibody onto the surface antibody bound to the antigen. Bind, intermediate and not bind categories were defined based on above 25% Rmax, 10-25% Rmax and below 10% Rmax, respectively.*

Conclusion

Alto was able to successfully characterize Sino Biological's highly specific antibodies and high-quality antigens by conducting kinetics and epitope analysis studies with DMF-powered SPR. Compared to traditional methods of characterization, SPR accelerates analysis of multiple interactions by eliminating the need for labels and reducing time-consuming preparation steps. The data presented here was acquired in <1 week, highlighting the efficiencies gained from Alto's ultra-low sample consumption coupled with the use of high quality reagents, such as the amine coupling kit used in this application, and automated on-cartridge serial dilutions. Alto's high-throughput capabilities can accelerate the discovery process by generating full kinetic analysis data and epitope binning studies from only 2 μl of sample, while requiring <20 min of hands-on time per experiment.

Appendix

Table 1: Sino Biological anti-NP and anti-HA antibodies (ligands).

	Name	Cat #
NP I	Pan Influenza A Nucleoprotein Antibody, Mouse MAb	40205-MM16
NP II	Pan Influenza A Nucleoprotein Antibody, Mouse MAb	40205-MM18
NP III	Pan Influenza A Nucleoprotein Antibody, Rabbit MAb	40205-R063
NP IV	Pan Influenza A Nucleoprotein Antibody, Rabbit MAb	40208-R014
NP V	Pan Influenza A Nucleoprotein Antibody, Rabbit MAb	40208-R017
HA I	Influenza A H3N2 (A/Brisbane/10/2007) Hemagglutinin / HA Antibody, Rabbit MAb	11056-R014
HA II	Influenza A H3N2 Hemagglutinin / HA Antibody, Rabbit PAb	11056-RP01

Table 2: Sino Biological recombinant proteins (analytes).

	Name	Cat #
Antigen NP 1	Influenza A H1N1 (A/California/07/2009) Nucleoprotein / NP Protein (His Tag)	40205-V08B
Antigen NP 2	Influenza A H3N2 (A/Hong Kong/1/1968) Nucleoprotein / NP Protein (His Tag)	40208-V08B
Antigen HA	Influenza A H3N2 (A/Brisbane/10/2007) Hemagglutinin / HA Protein (His Tag)	11056-V08B